

Determination of Aflatoxin M₁ and Ochratoxin A in Raw, Pasteurized and UHT Milk in Turkey

Cagla Turkoglu & Erhan Keyvan

ABSTRACT

Background: Mycotoxins produced by yeast and fungi have toxic effects on human and animal health. Aflatoxin B₁ (AFB₁) is the most toxic hepatocarcinogen to mammals. Aflatoxin M₁ (AFM₁), which has been found in milk and dairy products, is the hydroxylated metabolite of AFB₁. Aflatoxin M₁ is formed by the cytochrome P450 enzyme in the liver. Ochratoxin A (OTA) is synthesized by *Aspergillus* and *Penicillium* species. Ochratoxin A is known to cause teratogenic, immunotoxic, nephrotoxic and carcinogenic effects. Due to the potential harmful effects on human and animal health, OTA has also been receiving increased attention globally; however, there is limited information on the presence of OTA in milk and dairy products. The aim of this study was to determine how mycotoxins impact the hygienic quality of raw and heat-processed milk.

Materials, Methods & Results: In this study, a total of 105 milk samples were analyzed (35 raw, 35 pasteurized and 35 UHT) to identify AFM₁ and OTA in raw, pasteurized and ultra-high temperature processing (UHT) milk. The levels of AFM₁ were detected by using the enzyme-linked immunosorbent assay (ELISA). The milk samples were centrifuged in order to remove the fat content from the milk. After centrifugation, the upper cream layer was withdrawn with a pipette. The non-fat liquid portion was placed in wells at 100 µL for analysis. The concentration of AFM₁ in the milk samples was analyzed by AFM₁ test kit. The milk samples with AFM₁ levels greater than 50 ng/L were confirmed by using High-Performance Liquid Chromatography (HPLC). An Ochratoxin A Serum / Milk ELISA test kit was used for the analyses of OTA. The analyses were made according to the manufacturer's instructions, and samples were analyzed in duplicate. The absorbance value of milk samples was obtained from the ELISA plate reader at 450 nm. The mean value of AFM₁ was found to be 19.54 ng/L in the milk samples. According to the European Commission (EC), the maximum limit for AFM₁ in milk is 50 ng/L. In our study, eight (7.61%) of the 105 samples exceeded this limit. The mean value of OTA was found to be 119 ng/L in the milk samples. The relationship between milk type and levels of AFM₁ was found to be significant at ($P < 0.01$). The mean value of AFM₁ in pasteurized milk was found statistically significant and lower than raw milk ($P < 0.05$). The difference between levels of OTA and milk type was not statistically significant at ($P > 0.05$).

Discussion: Milk is a great protein source especially for children in the age of growth. Yeasts such as *Fusarium*, *Aspergillus* and *Penicillium* produce mycotoxins that cause food, feed contamination. Owing to carcinogenic, mutagenic and teratogenic effects of AFM₁, presence of AFM₁ in milk samples may adversely affect human health. The presence of AFM₁ in different contamination levels can be observed in milk and milk products. Factors such as ration type, climate conditions, feed storage conditions, feeding regime and health status of dairy animals may be effective in the occurrence of these contamination. It is necessary to establish legal limits by conducting effective research on the existence of OTA in animal-derived products. The existence of mycotoxins in milk and dairy products can be reduced by preventing the contamination of feed materials with yeast and molds used in the feeding of dairy cows. Milk is one of the most important protein source for the human, effective hygienic controls should be applied to prevent microbiological and chemical hazards. Our data suggest that heat-treated milk may also be dangerous to human health, mycotoxins contamination should be controlled with monitoring programs routinely in milk and feed materials for food safety.

Keywords: aflatoxin M₁, ochratoxin A, Milk.

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Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Burdur, Turkey. CORRESPONDENCE: E. Keyvan [erhankeyvan@mehmetakif.edu.tr - Fax: +90 (248) 213 20 00]. Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, 15030 Burdur, Turkey.

INTRODUCTION

Aflatoxins (AF) have hepatotoxic, carcinogenic and immunosuppressive effects which are generally produced by strains of *Aspergillus flavus* [40,49]. Aflatoxin M₁ (AFM₁) is the hydroxylated metabolite of aflatoxin B₁ (AFB₁) which has been found in milk and dairy products [12]. Aflatoxin M₁ shows resistance to heat, and milk processing technologies, like pasteurization and sterilization, cannot deactivate it [6]. It is also possible for milk products, like yoghurt, butter and cheese, to be contaminated with AFM₁ because of milk contamination [8,11,21]. In recent years, many researchers have reported AFM₁ contamination in raw, pasteurized and Ultra High Temperature (UHT) milk from Turkey and other countries [5,7,30,31,45] (Table 1). The potential existence of AFM₁ levels in milk and milk products

is the reason they should be monitored continuously for the sake of public health [22].

Ochratoxin A (OTA) is a mycotoxin which contaminates a wide range of plant products [4,27,33,46]. Ochratoxin A was classified into group 2B as a possible human carcinogen [27]. Even though it originates in plant products, OTA is a stable molecule that can remain unchanged in food even after processing [43]. Carryover contamination of OTA was observed in pigs and chickens fed with feed containing low levels of OTA [34,35]. Moreover, high levels of OTA contamination were found in food in Europe [50]. Consumption of OTA-contaminated, animal-derived food products are a public health risk. [1]. For this reason, it is important for meat and milk to be tested and monitored for OTA content [16]. The objectives of this study were to determine AFM₁ and OTA content in raw, pasteurized and UHT milk samples.

Table 1. Incidence of AFM₁ from different countries.

Milk type	Sample no.	Positive sample no.	AFM ₁ (ng/L)	Exceed 50 ng/L* (%)	Country	Ref.
Raw and pasteurized	180	100 (55.6%)	21.31	30 (30.0)	Iran	[23]
Raw	254	204 (80.3%)	66.00	144 (56.7)	Iran	[19]
Raw	3,635	1538 (42.4%)	14.3	105 (2.9)	Macedonia	[14]
Raw	129	129 (100%)	19.50	18 (19.95)	Brazil**	[39]
Raw and UHT	194	47 (24.23%)	20.60	13 (27.66)	Crotia	[7]
Pasteurized	25	17 (68.00%)	30.60	4 (23.53)	Lebanon	[6]
Raw	92	5 (5.43%)	20.50	0 (0.00)	Spain	[41]
Raw	678	540 (79.65%)	282.00	382 (70.74)	Serbia	[45]
Raw	416	51 (12.3%)	37.00	0 (0.00)	Italy	[42]
Raw	5,650	267 (4.7%)	36.8	63 (1.1)	China**	[31]
Raw	156	143 (91.7%)	346.2	125 (80.1)	Pakistan	[5]
UHT	41	30 (91.1%)	17.76	3 (7.3)	Turkey	[30]

*European Commission and Turkish Food Codex determined the limit of AFM₁ in milk as 50 ng/L. **Brazil and China determined the limit of AFM₁ in milk as 500 ng/L.

MATERIALS AND METHODS

Milk samples

In our study, a total of 105 milk samples were analyzed (35 raw, 35 pasteurized and 35 UHT) to identify AFM₁ and OTA in raw, pasteurized and UHT milk.

ELISA Analysis

The milk samples were centrifuged at 3,500 g for 10 min at 10°C (Eppendorf 5804R, Germany)¹ in order to remove the fat content from the milk. After centrifugation, the upper cream layer was withdrawn

with a pipette. The non-fat liquid portion was placed in wells at 100 µL for analysis. The concentration of AFM₁ in the milk samples was analyzed by competitive enzyme-linked immunosorbent assay (ELISA) using an AFM₁ test kit (RIDASCREEN; R-Biopharm, Darmstadt, Germany)² procedure suggested by the manufacturer, with a limit of detection (LOD) of 5 ng/L for milk. An Ochratoxin A Serum / Milk ELISA (Helica, 9910CH01MS-96)³ test kit was used for the analyses of OTA. The analyses were made according to the manufacturer's instructions, and samples were analyzed in duplicate.

The absorbance value of milk samples was obtained from the ELISA plate reader at 450 nm (ELX-800; Bio-Tek Instruments, Winooski, VT, USA)⁴ and RIDAWIN software (RIDA SOFT Win; R-Biopharm AG)² was used to evaluate the results. The samples containing more than 5 ng/L of AFM₁ were considered positive assays. The standart curve in Figure 1 was used to determine OTA values.

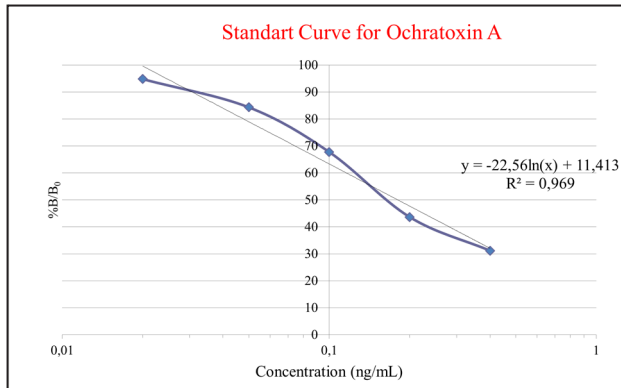


Figure 1. Typical standard curves ($y = -22,56\ln(x) + 11,413$ $R^2 = 0,969$) for ochratoxin A used in the evaluation of Ochratoxin A ELISA ($n = 105$). B/B₀, ratio of the absorbance corresponding to the given standard to the absorbance of the 0 ng/mL standard. (maximum signal).

HPLC Analysis

The milk samples with AFM₁ levels greater than 50 ng/L were confirmed by using High-Performance Liquid Chromatography (HPLC) in the Scientific and Technology Application and Research Center of Mehmet Akif Ersoy University. For the measurements of AFM₁ in the samples, the method of Dragacci *et al.* [15] was adopted for HPLC. After reaching a temperature of 37°C, the fat from the milk samples (100 mL) was removed by centrifugation at 2,000 xg. Defatted milk was filtered through Whatman No. 4 filter paper. Fifty milliliters of filtrated milk was passed through an immunoaffinity clean-up column (VICAM, USA)⁵. The column was washed with 20 mL of distilled water and then air dried. Aflatoxin M₁ was eluted with 4 mL of acetonitrile, and the acetonitrile phase was evaporated. The residue was dissolved in 3 mL of mobile phase and was injected into the HPLC system (Shimadzu, Kyoto, Japan)⁶.

Statistical Analysis

The difference between the milk sample types and AFM₁ and OTA levels (see in Table 2) was determined by one-way analysis of variance (ANOVA) and

Tukey's test using the SPSS software package (version 15.0 for Windows)⁷.

RESULTS

In this study, a total of 105 milk samples (35 raw, 35 pasteurized and 35 UHT) were analyzed to detect contamination levels of AFM₁ and OTA. Table 2 shows the levels of AFM₁ and OTA contamination in the milk samples. The range of AFM₁ contamination in raw, pasteurized and UHT milk samples was found to have a mean value of 25.45 ± 3.38 ng/L, 12.86 ± 1.05 ng/L, 20.29 ± 2.77 ng/L, respectively. The mean levels of OTA contamination were detected at 137 ± 57 ng/L, 135 ± 8 ng/L, 85 ± 4 ng/L in raw, pasteurized and UHT milk samples, respectively. According to the results, AFM₁ levels in eight of the 105 (7.61%) milk samples were determined higher than the maximum limit of the European Commission (EC) [18] (Table 3). The mean value of AFM₁ in pasteurized milk was found statistically significant and lower than raw milk ($P < 0.05$). The difference between levels of OTA and milk type was not statistically significant at ($P > 0.05$).

DISCUSSION

The presence of AFM₁ in milk and milk products is a significant public health problem, as AFB₁ and AFM₁ are human carcinogens [27,33]. In the current study, nearly all milk samples were found to be positive for signs of AFM₁ (limit of detection: 5 ng/L). High levels of AFM₁ have also been reported in Turkey, Iran, Pakistan and Kuwait [2,10,13,24,37]. By contrast, researchers from Turkey, Croatia, Iran and Macedonia reported lower levels of AFM₁ than the current study [7,14,23,30].

Various levels of AFM₁ can contaminate milk and dairy products. Contamination differences can arise from multiple factors, such as ration type, climate conditions, feed preservation conditions, feeding regime, and the health statuses of dairy animals. In Dimitrieska-Stojkovic *et al.*'s [14] study, they determined an AFB₁ contamination rate of 13.4% in feed materials, estimating that the carryover levels of AFM₁ in milk ranged from 0.22% to 3.47%. In summary, this study revealed that AFB₁ contamination in feed materials can act as a significant source of AFM₁ in milk. Contamination of milk and dairy products with AFM₁ is a serious problem for public health. AFM₁ should be routinely monitor as a food quality control measure for human health [38].

Table 2. AFM₁ and OTA mean values in raw, pasteurized and UHT milk.

Type of milk sample	Mean AFM ₁ (ng/L) ± SE ¹	Mean OTA (ng/L) ± SE
Raw	25.45 ^a ± 3.38	137 ^a ± 57
Pasteurized	12.86 ^b ± 1.05	135 ^a ± 8
UHT	20.29 ^{ab} ± 2.77	85 ^a ± 4
Total	19.54 ± 1.57	119 ± 19

^{a,b,c}The difference between groups with different letters in the same column is statistically significant at (*P* < 0.05). ¹The relationship between milk type and levels of AFM₁ was found to be significant at (*P* < 0.01).

Table 3. Occurrence of AFM₁ in raw, pasteurized and UHT milk.

Type of milk sample	Samples tested	Positive samples* (%)	Exceed 50 ng/L (%)
Raw	35	35 (100)	5 (14.28%)
Pasteurized	35	35 (100)	0 (0%)
UHT	35	34 (97.14)	3 (8.57%)
Total	105	104 (99.04)	8 (7.61%)

*Containing >5 ng/L AFM₁.

The level of AFM₁ contamination in milk and dairy products may depend on many factors, such as geographical location, seasonal effects and milk type [28]. Based on our results, the mean value of AFM₁ contamination was found to be 19.54 ng/L in all milk samples. All the same, contamination levels of AFM₁ in milk samples from Pakistan, Serbia, Iran, and Lebanon have had varied values of 346.2 ng/L, 282.0 ng/L, 66.0 ng/L, and 30.60 ng/L, respectively [5,6,19,45]. Therefore, because the high-consumption level of AFM₁-contaminated milk and dairy products poses serious health problems, national authorities should enroll in educational programs about farm management and feed contamination.

Seasonal differences can also affect the degree of AFM₁ contamination in collected samples. Previous studies have shown that levels of AFM₁ were higher in winter seasons than in warmer months of the same year [20,26]. Therefore, the concentrated feeding of dairy cows during winter seasons may contribute to AFM₁ contamination in milk. As the present study does not provide a seasonal assessment, subsequent studies about the prevalence of AFM₁ in milk and dairy products should evaluate the influence of seasonal factors, as well.

The European Commission and Turkish Food Codex determined the limit of AFM₁ in milk to be 50 ng/L [18,45]. All the same, in the current study, 14.28% of the raw milk samples and 8.57% of the UHT were found to have higher values than the Turkish Food Codex's maximum AFM₁ limit. Due to AFM₁ regulations of China (500 ng/L), Xiong *et al.* [51] reported lower excess levels of AFM₁ in UHT milk

samples than the current study. Pei *et al.* [38] were reported that all of the samples were below the Chinese limit, while 72% (97 out of 135) milk products samples were found to be higher than the maximum acceptable limits (50 ng/L) of the EU. Studies from different countries were reported various levels of AFM₁ depending on the legislative differences. Even if the obtained results are below the relevant legal regulations, the effect of long term AFM₁ exposure on humans should be investigated.

Despite, none of the pasteurized milk samples exceeded the AFM₁ limit, and the mean levels of the samples were found to be 12.86 ng/L in the present study. Prolonged exposure to aflatoxin in small quantities can cause cancer and immunosuppression [32]. Thermal processes, such as pasteurization and sterilization, do not affect AFM₁ [6]. Therefore, heat-treated milk may also be dangerous to human health.

Ochratoxins are found in a wide range of foods and feedstuffs. More specifically, OTA can be identified in animal-derived products, such as meat and milk [3]. A few studies have studied the prevalence of OTA in milk. In the current study, the mean OTA levels in milk samples were observed to be higher than in previous research (see in Table 4). While the EC established a limit for OTA in foodstuffs, outside of Slovakia, which restricts the amount of OTA in milk to 5 µg/kg, there are no regulations to reduce the incidence of OTA in milk and milk products. By comparison, the current study found amounts of OTA that were lower than the limits established in Slovakia.

Table 4. Ochratoxin A in milk.

Type of milk sample	Sample no.	Positive samples	Contamination Level (ng/L)	Ref.
Conventional milk sample	40	6	11-58	[44]
Organik milk sample	47	5	15-28	[44]
Cow's milk sample	36	5	10-40	[9]
Cow's milk sample	121	ND	ND	[48]
Bulk tank milk sample	40	37	10-270	[29]

*ND: Non detected.

In a previous work, we calculated the maximum limits of OTA (200 ng/L) for a child (four-years old) in accordance with the Nordic Working Group [36], which suggested a tolerable daily intake (TDI) of 5 ng/kg bw of OTA in humans [29]. In the current study, 4 out of 105 (3.8%) milk samples showed more than the maximum limit of 200 ng/L of OTA. The European Food Safety Authority (EFSA) established the tolerable weekly intake (TWI) of OTA to be 120 ng/kg bw [19]. According to the results of the current study, the weekly tolerable OTA level was below the limit that was reported by the EFSA. As such, additional research should work to assess OTA's presence within milk and dairy products. Although there are no limitations or legislation about the presence of OTA in milk and milk products, small quantities of OTA can prove harmful, especially to children.

CONCLUSION

Because milk is one of the most important sources of protein for human beings, effective hygienic controls should be applied to prevent microbiological and chemical hazards. To ensure food safety, mycotoxin contamination should also be routinely controlled with monitoring programs of milk and feeding

materials. Due to the carcinogenic, mutagenic, and teratogenic effects of AFM₁, its presence in dairy can adversely influence human health. As adults have access to a more diverse diet than infants and children, OTA in cow's milk is also more likely to impact the young. It is therefore necessary to establish legal limits by conducting effective research on the existence of OTA in animal-derived products. Mycotoxins can also be reduced in milk and dairy products by avoiding the use of feeding materials that have been contaminated with yeast and molds.

MANUFACTURERS

¹Eppendorf AG. Hamburg, Germany.

²R-Biopharm AG. Darmstadt, Germany.

³Helica Biosystems Inc. Santa Ana, CA, USA.

⁴Bio-Tek Instruments Inc. Winooski, VT, USA.

⁵VICAM. Milford, MA, USA.

⁶Shimadzu Corp. Kyoto, Japan.

⁷SPSS Inc. Chicago, IL, USA.

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